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Collaborative Comparison of Broth Macrodilution and Microdilution Antifungal Susceptibility Tests

A. ESPINEL-INGROFF,[1]* C. W. KISH, JR.,[1] T. M. KERKERING,[1] R. A. FROMTLING,[2] K. BARTIZAL,[3] J. N. GALGIANI,[4] K. VILLAREAL,[4] M. A. PFALLER,[5]† T. GERARDEN,[5] M. G. RINALDI,[6] AND A. FOTHERGILL[6]

Medical College of Virginia, Richmond, Virginia 23298-0049[1]; Science and Technology Policy, Merck & Co., Inc.,[2] and Merck Research Laboratories,[3] Rahway, New Jersey 07065; Veterans Affairs Medical Center and University of Arizona College of Medicine, Tucson, Arizona 85723[4]; University of Iowa College of Medicine, Iowa City, Iowa 52242[5]; and University of Texas Health Sciences Center, San Antonio, Texas 78284[6]

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A collaborative comparison of macro- and microdilution antifungal susceptibility tests was performed in five laboratories. MICs of amphotericin B, fluconazole, flucytosine, and ketoconazole were determined in all five centers against 95 coded isolates of *Candida* spp., *Cryptococcus neoformans*, and *Torulopsis glabrata*. A standard protocol with the following National Committee for Clinical Laboratory Standards Subcommittee on Antifungal Susceptibility Testing recommendations was used: an inoculum standardized by spectrophotometer, buffered (RPMI 1640) medium (pH 7.0), incubation at 35°C, and an additive drug dilution procedure. Two inoculum sizes were tested (1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml) and three scoring criteria were evaluated for MIC endpoint determinations, which were scored as 0 (optically clear), ≤1 (slightly hazy turbidity), and ≤2 (prominent decrease in turbidity compared with that of the growth control). Overall intra- and interlaboratory reproducibility was optimal with the low-density inoculum, the second-day readings, and MICs scored as either 1 or 2. The microdilution MICs demonstrated interlaboratory agreement with most of the four drugs higher than or similar to that of the macrodilution MICs. In general, there was good interlaboratory agreement with amphotericin B, fluconazole, and flucytosine; ketoconazole gave more variable results.

The incidence of fungal infections, especially yeast infections, has increased greatly because of the frequent use of cytotoxic and antibacterial drugs (31). This incidence is even greater among patients infected with the human immunodeficiency virus (HIV) (1, 12, 14, 16). The higher incidence of fungal infections has increased the use of antifungal agents, especially the numerous imidazoles (28, 30). The impact of *Candida* infections is substantial, with the attributable mortality and excess length of hospitalization being estimated at 30% and 30 days (median value), respectively (31). Infections such as oral candidiasis rarely become life-threatening diseases in AIDS patients; however, 36 to 85% of HIV-infected patients suffer from *Candida* infections (1, 7, 12, 14, 16). The recurrence of both vaginal and oral infections among AIDS and non-AIDS patients has been reported more frequently in the last few years (1, 7, 12, 14, 16, 27). This recurrence has been associated with the presence of different biotypes of *Candida albicans* for each episode of oral infection (1) and/or the switching of colony phenotypes for vaginal infections (27). Furthermore, contradictory reports that biotyping changes are accompanied by antifungal susceptibility changes exist, and the development of antifungal resistance with prolonged treatment also has been reported (7, 13, 16, 18, 26, 27). However, susceptibility testing with antifungal agents, in general and especially with azoles, against yeast cells is vastly influenced by medium composition, length of incubation and temperature, and inoculum size (3, 4, 9, 15, 17, 29).

The broth macrodilution test is the most widely used technique for antifungal susceptibility testing (25). It also permits the determination of minimum fungicidal (lethal) concentrations. The need to develop standardized reference methods for antifungal susceptibility tests has been perceived previously (10, 19), and progress has been made in this direction by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests. Preliminary recommendations for broth macrodilution susceptibility testing of yeasts have been provided as a result of two collaborative studies by the Subcommittee (22, 23). These recommendations include the use of the chemically defined tissue culture medium RPMI 1640 buffered with morpholinopropanesulfonic acid (MOPS) (pH 7.0), inocula standardized by a spectrophotometric method, and incubation at 35°C for 24 to 48 h for testing with flucytosine, amphotericin B, and ketoconazole. However, the widely used agent fluconazole (28, 30), now commercially available, was not included (23). The evaluation of the microdilution antifungal test also has not been addressed by the NCCLS Subcommittee. A preliminary comparison study of both broth macro- and broth microdilution antifungal tests in which the NCCLS Subcommittee recommendations were followed already has been reported (6). The latter study showed that, with a few exceptions, discrepancies between the two tests were not significant and that it may be possible to utilize the more efficient and economical broth microdilution test, which is also easier to perform, in the clinical laboratory.

The purpose of this collaborative (five-center) study was to evaluate the broth microdilution antifungal susceptibility test and to compare it with the broth macrodilution test. This study was performed simultaneously with the NCCLS multicenter (13-center) study of broth macrodilution antifungal testing with amphotericin B, flucytosine, and ketoconazole,

* Corresponding author.
† Present address: Department of Pathology, Oregon Health Sciences University, Portland, OR 97201.

to be reported elsewhere (8). In our study, fluconazole also was evaluated by both methods, in addition to the other three antifungal agents. The drug dilutions, inocula, media, and reading criteria used were the same for both tests, as were the two inoculum densities: 1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml.

MATERIALS AND METHODS

Study design. Five laboratories (coded as laboratories 1, 2, 3, 4, and 5) participated in this study, and each laboratory received the same 100 coded test isolates from the University of Iowa. Each laboratory evaluated each isolate with the four antifungal agents by both broth micro- and broth macrodilution antifungal susceptibility tests following a standard protocol. This protocol included the NCCLS Subcommittee recommendations for the broth macrodilution testing of yeast cells (23); two inoculum densities were evaluated by each laboratory by both techniques. Tubes and wells were scored on a scale of 1 to 4; scores were recorded on standardized data sheets supplied to each laboratory by the University of Arizona College of Medicine.

The objectives of this study were (i) to determine the intralaboratory variability between broth micro- and macrodilution MIC endpoints with identical prepared drugs, media, inoculum sizes, incubation times, and reading criteria, (ii) to determine the interlaboratory reproducibility of both broth macro- and broth microdilution MIC endpoints, (iii) to determine the correlation of broth macro- and microdilution MIC endpoints when three different MIC endpoint-scoring criteria are evaluated, and (iv) to evaluate the effects of two inoculum densities, 1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml, by both tests.

Antifungal drugs. MICs of amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), fluconazole (Roerig-Pfizer, New York, N.Y.), flucytosine (Hoffmann-LaRoche Laboratories, Inc., Nutley, N.J.), and ketoconazole (Janssen Pharmaceutica, Piscataway, N.J.) were determined by broth macro- and microdilution techniques. The four drugs were supplied by the manufacturers as standard powders from the same lot and were distributed to each participant laboratory. Amphotericin B and ketoconazole were dissolved in 100% dimethyl sulfoxide to obtain stock solutions of 16,000 μg/ml, with the weight adjusted according to the potency of each drug. Stock solutions of flucytosine and fluconazole (each 16,000 μg/ml), adjusted to their respective provided potencies, also were prepared with sterile distilled water.

Cultures. A panel of 100 well-characterized clinical isolates of pathogenic yeasts was sent, as coded strains, to each laboratory. One of the laboratories did not test five isolates (one isolate each of *C. albicans*, *C. tropicalis*, and *Torulopsis glabrata* and two *C. neoformans* isolates); therefore, these five isolates were not included in the analysis of the data. The 100 isolates included 35 isolates of *C. albicans*, 15 isolates each of *C. parapsilosis*, *C. tropicalis*, and *C. neoformans*, and 10 isolates each of *C. lusitaniae* and *T. glabrata*. The *Candida* spp. and *T. glabrata* isolates were from the culture collection at the University of Iowa, and the 15 isolates of *C. neoformans* were from the collection at the Medical College of Virginia. Each isolate represented a unique isolate from a patient and was maintained as a water suspension at room temperature in each laboratory until testing was performed. The same isolate of *C. parapsilosis* also was provided to each center to be included as the control each time that a group of isolates was tested by both methods. This quality control (QC) isolate was tested previously, and it demonstrated antifungal MICs within the test ranges of each of the four drugs tested (<1.0 μg/ml) (6).

Procedure. The standard protocol provided to each laboratory contained detailed instructions concerning the preparation of the twofold drug dilutions, the inoculum densities, the step-by-step procedure, and the scoring of MIC tubes and wells.

(i) Medium. Liquid RPMI 1640 (RPMI) medium with both L-glutamine and 0.165 M MOPS buffer (34.54 g/liter) and without sodium bicarbonate was provided (ready for use) to all participants by ICN Biomedicals, Inc. (Costa Mesa, Calif.) and Whittaker Bioproducts, Inc. (Walkersville, Md.). The pH of both media was 7.0 at 35°C. Sterility control of each bottle was performed before it was used. Laboratories 1 and 3 were able to test all isolates using the RPMI 1640 from ICN, and the other three laboratories used both RPMI media (ICN and Whittaker).

(ii) Drug dilutions. Both broth macro- and broth microdilution tests were performed by using the same modified NCCLS standard (approved M7-A2) additive twofold drug dilution schema to reduce pipetting errors (20). The broth macrodilution test drug dilutions were prepared to be 10× the strength of the final drug concentration, with medium as the diluent (e.g., 640 to 1.20 μg/ml for fluconazole and flucytosine and 160 to 0.3125 μg/ml for amphotericin B and ketoconazole).

For the microdilution tests, each 10× drug dilution was further diluted 1:5 in medium to provide the 2× strength needed for the test.

(iii) Preparation of stock inoculum suspensions. All isolates were subcultured at least twice in each laboratory to ensure purity and viability. The *Candida* spp. and *T. glabrata* isolates were grown on Sabouraud dextrose agar (Difco, Detroit, Mich.) for 24 h at 35°C. The isolates of *C. neoformans* were grown on the same agar for 48 h at 35°C. Yeast cells from at least five 1-mm-diameter colonies from the 24- or 48-h-old cultures were suspended in 5 ml of sterile 0.85% saline. The resulting yeast suspension was mixed for 15 s with a vortex. The turbidity of each mixed suspension was measured at 530 nm and adjusted to 1×10^6 to 5×10^6 CFU/ml by the spectrophotometric method previously described (22). The final transmission of each yeast suspension ranged from 75 to 77%. Quantitation of the test inoculum to obtain the number of CFU per milliliter was performed by using quantitative colony plate counts of the cell suspensions as described below.

(iv) Preparation of both test inocula. Two test inocula were evaluated by each test: 1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml.

(a) Inocula for macrodilution test. The adjusted stock yeast suspensions were diluted 1:100 with RPMI 1640 medium to obtain the first final desired test inoculum size of approximately 1×10^4 to 5×10^4 CFU/ml. The yeast suspension(s) was mixed for 15 s prior to each dilution step. This suspension(s) was further diluted 1:20 for the 0.5×10^3 to 2.5×10^3 CFU/ml (second) test inoculum. Both test inocula were made in sufficient volumes to directly inoculate each MIC tube with 0.9 ml.

(b) Inocula for microdilution test. The following dilutions were performed to prepare the two test inocula for the microdilution test. The adjusted stock yeast suspensions were diluted 1:50 for the highest test inoculum and further diluted 1:20 for the lowest test inoculum. The cell suspension(s) was mixed for 15 s before each dilution step with a vortex. The two diluted suspensions resulted in inoculum sizes of approximately 0.2×10^5 to 1×10^5 and 1×10^3 to 5

$\times 10^3$ CFU/ml, respectively, or 2× the final desired test inocula. The 2× inoculum was diluted 1:1 when the wells were inoculated, and the desired final inoculum sizes were achieved.

(v) Confirmation of inoculum sizes. The confirmation of inoculum sizes was determined with the final higher test inoculum for both broth macro- and broth microdilution tests. These diluted yeast suspensions were mixed for 15 s with a vortex, and 0.001-ml volumes of the former suspension and 0.01-ml volumes of the latter suspension were removed with a calibrated loop. Each volume was spread onto a Sabouraud dextrose agar plate with a sterile, bent glass rod. Plates were incubated at 35°C until growth was evident, and the CFU per milliliter were determined. This allowed counts of 10 to 50 colonies per plate.

(vi) Broth macrodilution test. The 10× drug dilutions were dispensed in 0.1-ml volumes into round-bottom, polystyrene, snap-cap, sterile tubes (12 by 75 mm) (Falcon 2054; Becton Dickinson Labware, Lincoln Park, N.J.). These drug dilutions were stored at −70°C for up to 1 month or overnight at 4 to 6°C before being tested. The tubes that were stored at −70°C were sealed with paraffin tape and, after being thawed, checked for dryness before being tested further. The day of the test, each tube was inoculated by adding 0.9-ml volumes of the corresponding diluted yeast inoculum suspension. This step brought the drug dilutions to the final test drug concentrations (16 to 0.03125 μg/ml for amphotericin B and ketoconazole and 64 to 0.125 μg/ml for fluconazole and flucytosine). The growth control tube(s) contained a 0.9-ml volume(s) of an inoculum suspension(s) and a 0.1-ml volume(s) of drug-free medium. The QC organism was tested in the same manner as the other isolates and was included each time that a set of isolates was tested in each laboratory by using both inoculum densities. In addition, 1 ml of uninoculated, drug-free medium was included as a sterility control.

(vii) Broth microdilution test. The broth microdilution tests were performed as previously described (6) by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells) (Dynatech Laboratories, Inc., Alexandria, Va.). The 2× drug concentrations were dispensed into the wells of rows 1 to 10 of the microdilution plates in 100-μl volumes with a multichannel pipette. Row 1 contained the highest drug concentration, and row 10 contained the lowest drug dilution. The microdilution plates were stored inside plastic bags at −70°C or overnight at 4 to 6°C. Each well was inoculated on the day of the test with 100 μl of the corresponding 2× diluted yeast inoculum suspension. This step brought the drug dilutions and inoculum densities to the final test concentrations mentioned above. The growth control wells contained 100 μl of sterile drug-free medium and were inoculated with 100 μl of the corresponding diluted inoculum suspension(s). The QC organism also was tested in the same manner and was included each time that a set of isolates was tested in each laboratory by using both inoculum densities. The wells of row 11 contained 100 μl of uninoculated, drug-free medium as a sterility control.

(viii) Incubation and scoring of MIC tubes and/or wells. All tubes and microdilution plates were incubated at 35°C and observed for the presence or absence of visible turbidity or growth at 24 and 48 h. When a lack of growth precluded the scoring of tubes and/or wells at 24 h, the tubes and/or microtiter plates were incubated again until visible growth permitted two consecutive scorings of tubes and/or wells (days 1 and 2). Each tube was flicked immediately prior to being scored, and its turbidity was compared with that of the growth control (drug-free) tube, which allowed the detection of small amounts of growth. The broth microdilution wells were scored with the aid of a reading mirror (Cooke Engineering Co., Alexandria, Va.); the growth in each well also was compared with that of the growth control (drug-free) well. A numerical score, which ranged from 0 to 4, was given to each tube and well by using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity (20). Each laboratory recorded the scores of the MIC tubes and wells on the standardized working sheets, which were mailed to the coordinator (Medical College of Virginia), where the results were entered into a computer data base.

(ix) Fluconazole MIC scores and growth control dilutions. A selected group of 15 *Candida* spp. was retested in four laboratories in order to correlate broth macrodilution MICs scored as 1 and 2 with 1:10 and 1:5 dilutions of the growth controls, respectively. These tests were performed by using a low-density inoculum, two growth control tubes, and the other testing conditions listed previously. Each day of reading, one of the growth controls was diluted 1:10 (0.1 plus 0.9 ml) and 1:5 (0.2 plus 0.8 ml), which are the equivalents of 90 and 80% inhibition, respectively. By using these tubes for comparison, the MIC with a 90% inhibition (MIC 90%) was defined as the lowest drug concentration with turbidity less than or equal to that of the 1:10 dilution, and the MIC 80% was defined as the lowest drug concentration with turbidity less than or equal to that of the 1:5 dilution. Comparison of the 60 MICs scored in this manner demonstrated 93% agreement among the four laboratories.

(x) Analysis of the data. Each of the 95 isolates was tested by both methods with the two densities and the four drugs, and each had two readings, one on day 1 and one on day 2. Each MIC endpoint was determined three ways by using a computer program as follows: the lowest drug concentration (tube and/or well) which had a score of 0 (optically clear), that which had a score of 1 (slightly hazy) or less, and that which had a score of 2 (prominent decrease in turbidity) or less were determined. Therefore, each yeast or isolate had 96 MIC scores per laboratory. Both on-scale and off-scale results were included in the analysis. The high off-scale MICs (>64 and >16 μg/ml) were converted to the next highest concentration (either 128 or 32 μg/ml), and the low off-scale MICs (≤0.12 and ≤0.03 μg/ml) were left unchanged. When skips (uneven patterns) were present, the MIC endpoint was the higher drug concentration. The listings of MIC endpoints were returned to the participant laboratories, where the accuracy was checked and errors were corrected. Discrepancies between MIC endpoints of no more than 2 dilutions (two tubes or wells) were used to obtain the percent values. The frequency of reproducibility, both intralaboratory (between both methods) and interlaboratory (among the five centers), was determined for each parameter and with the three MIC scores across species and by species.

RESULTS

Candida spp. and *T. glabrata* isolates produced clearly detectable growth at 24 h in the RPMI 1640 medium, and the first determination of MIC endpoints was possible at that time in the five laboratories. The MIC endpoints of *C. neoformans* isolates could not be determined at 24 h with all isolates with the low-density inoculum in most laboratories (four of five laboratories). The actual inoculum sizes of the

TABLE 1. Interlaboratory agreement for broth macro- and microdilution antifungal tests for 95 pathogenic yeast isolates[a]

Drug	Test condition[b]	% Agreement[c]					
		Micro			Macro		
		0	1	2	0	1	2
Amphotericin B	H/24	96	96	94	84	90	85
	H/48	96	97	96	84	92	90
	L/24	97	97	95	87	86	85
	L/48	99	99	95	90	93	91
Fluconazole	H/24	85	82	84	79	80	87
	H/48	90	89	76	82	86	80
	L/24	85	82	89	77	74	93
	L/48	89	87	79	86	81	88
Flucytosine	H/24	84	92	94	76	91	94
	H/48	86	87	94	79	88	93
	L/24	89	94	96	89	93	96
	L/48	90	92	94	90	93	94
Ketoconazole	H/24	82	74	69	77	70	82
	H/48	87	81	76	84	80	71
	L/24	75	69	80	76	68	89
	L/48	84	77	67	81	79	78

[a] Agreement is defined as the largest MIC subset with a range of ≤2 and is based on 22,800 MIC endpoints, including off-scale results.

[b] H and L, high- and low-density inocula (1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml, respectively); 24 and 48, first- and second-day readings, respectively.

[c] Micro and macro, micro- and macrodilution MICs, respectively. Scores were as follows: 0, optically clear; 1, slightly hazy turbidity; and 2, prominent decrease in turbidity.

TABLE 2. Intralaboratory comparison of broth micro- and macrodilution antifungal tests

Drug	Test condition[a]	% Median value agreement at[b]:		
		0	1	2
Amphotericin B	H/24	94	97	98
	H/48	93	96	94
	L/24	99	99	98
	L/48	98	97	95
Fluconazole	H/24	85	89	82
	H/48	86	91	71
	L/24	85	80	90
	L/48	92	88	77
Flucytosine	H/24	81	92	96
	H/48	79	89	93
	L/24	91	93	96
	L/48	90	95	97
Ketoconazole	H/24	77	81	80
	H/48	86	77	68
	L/24	78	72	81
	L/48	88	81	71

[a] H and L, high- and low-density inocula (1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml, respectively); 24 and 48, first- and second-day readings, respectively.

[b] Agreement of MIC endpoint pairs (≤2 dilutions) was scored as 0 (optically clear), 1 (slightly hazy turbidity), or 2 (prominent decrease in turbidity).

stock suspensions ranged from 1.2×10^6 to 4.8×10^6 CFU/ml for the 95 isolates tested in each center and also for the QC isolate included in each set (2 to 15 sets per laboratory) of tests performed.

Microdilution interlaboratory agreement: all MICs included. Table 1 represents the summary of interlaboratory reproducibility of the 22,800 microdilution MIC endpoints across the species for the 95 yeast isolates tested, stratified by antifungal agents, length of incubation, inoculum density, and MIC scoring criteria. The values are the percentages of total MICs in agreement out of the total microdilution MICs. For each set of MICs from the five sites, MICs were considered in agreement when they belonged in the largest MIC subset with a range of no greater than 2 dilutions. Excellent reproducibility (94 to 99%) was observed among the five laboratories with amphotericin B, regardless of inoculum size, incubation time, or endpoint criteria. Fluconazole MIC endpoints also showed similar but lower reproducibility with the different parameters (82 to 90%); the exceptions were second-day MICs scored as 2 (76 to 79%). The results with flucytosine and ketoconazole were more dependent on the different parameters. The highest reproducibility (96%) for flucytosine was observed when microdilution MICs were determined on day 1 with the low-density inoculum and were scored as 2; day 2 scores (94%) were next in reproducibility. On the other hand, ketoconazole MIC endpoints were less variable among the five laboratories when they were determined on the second day and scored as 0 (84 to 87%). The second-day scores provided higher reproducibility than the first-day scores with some drugs and under certain testing conditions (Table 1).

Macrodilution interlaboratory agreement: all MICs included. Table 1 also summarizes the interlaboratory agreement for the 22,800 macrodilution MICs. With the exception of fluconazole MICs, these results are a subset of the results reported elsewhere (8), but the percentages were obtained in the same manner described for the microdilution MICs. The pattern of reproducibility of macrodilution results was similar to that for the microdilution MICs. Fluconazole MICs showed the highest reproducibility on day 1 and when they were scored as 2 (90%); day 2 scores (88%) were next in reproducibility. Furthermore, the percent agreement among the five laboratories was lower than the percent agreement observed by the broth microdilution test with some of the four drugs (Table 1).

Micro- and macrodilution comparison. Forty-five thousand six hundred micro- and macrodilution MICs were used for the intralaboratory comparison of MIC pairs of the 95 yeast isolates tested, which included off-scale endpoints. For each combination of test conditions (e.g., inoculum density, drugs, and scoring criteria), macro- and microdilution MICs from each laboratory were considered in agreement when the discrepancies between the tests were within 2 dilutions. Only fluconazole and ketoconazole broth microdilution MICs scored as 2 were consistently higher than macrodilution MICs. Table 2 displays the median of percent agreement between the two methods in the five centers. There was good intralaboratory agreement in most laboratories for amphotericin B under all test conditions and scoring criteria and with flucytosine when the low-density inoculum was used and MICs were scored as 2. However, a comparison of fluconazole and ketoconazole MIC pairs demonstrated more variable results. Good (≥90%) intralaboratory reproducibility was obtained with fluconazole on day 1 with low-density inoculum MICs scored as 2 (also on day 2 with low-density inoculum MICs scored as 0 and with high-density inoculum MICs scored as 1). With ketoconazole, the best agreement

TABLE 3. Ranges for a QC isolate[a] with four antifungal drugs in five laboratories

Drug	MIC range (μg/ml)[b]	Testing conditions[c]	
		Macrodilution	Microdilution
Amphotericin B	0.5–2	48 h, L, 2	48 h, H, 0, 1 48 h, L, 0, 2
Fluconazole	≤0.12–0.5	24 h, 48 h, L, 1, 2	48 h, L, 2
Flucytosine	≤0.12–0.5	48 h, L, 2	24 h, 48 h, L, 1, 2
Ketoconazole	≤0.03–0.5	48 h, L, 2	48 h, L, 2

[a] The QC isolate (*C. parapsilosis*, Medical College of Virginia isolate no. 52.493) was tested by the broth microdilution test with all parameters 47 times and by the broth macrodilution test 31 times.

[b] 90 to 100% agreement among the five laboratories for the QC MIC endpoints.

[c] 24 h and 48 h, first and second readings, respectively; H and L, high- and low-density inocula (1×10^4 to 5×10^4 and 0.5×10^3 to 2.5×10^3 CFU/ml, respectively); 0, 1, and 2, MICs scored as 0 (optically clear), 1 (slightly hazy turbidity), or 2 (prominent decrease in turbidity).

between the two tests was on day 2 and with MICs scored as 0 (Table 2). Since most *C. neoformans* MICs were not determined at the same incubation times as those for the other yeasts, agreement between both tests also was analyzed with the scores for these isolates excluded, and the results were similar.

Interlaboratory agreement for the QC isolate. Each time a set of MICs (2 to 15 sets in each laboratory) was determined by either test, the QC isolate was introduced as a control of drug activity. The QC isolate MICs were within previously demonstrated ranges (6) with the four drugs in most laboratories (Table 3). Interlaboratory reproducibility (within ±1 dilution) of MIC endpoints (31 microdilution and 47 macrodilution replicates) for this QC isolate was dependent on the different parameters investigated. In general, there was better agreement with amphotericin B by the microdilution test with 48-h MICs scored as 0 or 2. On the other hand, more reproducible results were observed when MICs were scored as 1 and 2 after both 24 and 48 h of incubation by both methods with the other three drugs (Table 3).

Distribution of susceptibility according to MIC scores. In general, both analyses of the data, off-scale endpoints included and excluded, indicated greater consistency among the laboratories when MICs were read on the second day and with the low-density inoculum. However, the different percentages of agreement among the four drugs were dependent on the scoring criteria. The three scoring criteria also produced a considerable shift in the distribution of susceptibilities with fluconazole and ketoconazole against most of the species tested. There was a gradual and substantial shift from high MICs to low MICs for isolates of *C. albicans*, *C. tropicalis*, and *T. glabrata* and, to a lesser degree, for those of *C. lusitaniae* and *C. parapsilosis* (Fig. 1). This shift was evident as the MIC scores changed from the most strict score, 0 (optically clear), to a less stringent score, 2 (prominent decrease in turbidity), as seen in Fig. 1 with broth microdilution fluconazole MICs.

Fluconazole MIC scores and growth control dilutions. Comparison of the 60 fluconazole MICs defined as MIC 80% (lowest drug concentration with turbidity less than or equal to the 1:5 dilution of the respective growth control) demonstrated 93% agreement among the four laboratories. Similar results were obtained with fluconazole MIC 90% (≤1:10 dilution of the growth control).

DISCUSSION

The goal of this study was to compare micro- and macrodilution antifungal testing of yeasts. It was conducted as a separate and parallel collaborative study of the expanded evaluation of a broth macrodilution test performed by the NCCLS Subcommittee in order to develop a reference method (8). Because of that, we followed their standard procedure, which included buffered (MOPS) RPMI 1640 medium, a 35°C incubation temperature, and the same panel of 100 pathogenic yeast isolates. However, we also evaluated fluconazole, in addition to amphotericin B, flucytosine, and ketoconazole, by both tests. It has been demonstrated previously that buffered RPMI medium supports adequate growth of pathogenic yeasts for antifungal susceptibility testing (6, 23). In our study, this medium also produced visible growth at 24 and 48 h with the two inoculum densities with most of the species tested by both tests. The exception was *C. neoformans*. Therefore, it was not possible to determine the first-day MIC endpoints in four laboratories before 24 h with the latter isolates.

The actual inoculum sizes of the stock yeast suspensions, including that of the QC isolate, were within the expected range of 1×10^6 to 5×10^6 CFU/ml recommended by the NCCLS Subcommittee by the spectrophotometric method (22, 23). Even though other methods of quantitation of fungi also give reliable results (2, 5, 21), the spectrophotometric method has yielded consistent intra- and interlaboratory MIC endpoints in our study and in previous ones (6, 8, 23). Moreover, this is an easy, quick, and familiar method to most laboratory personnel. Consequently, this study supports the established NCCLS criteria for inoculum preparation, an important step in the development of antifungal susceptibility standards.

The determination of MIC endpoints is a critical step in antifungal susceptibility testing and is even more so with the azoles. The usual partial inhibition or trailing that is observed with the latter compounds precludes the determination of well-defined MICs. As in our study, this trailing effect previously has been scored as 1 (6, 23), providing an easier evaluation of the azoles and of flucytosine. However, the distributions of ketoconazole and fluconazole MICs scored as 1, for most *Candida* spp. and *T. glabrata* isolates, were uniformly higher, as demonstrated in Fig. 1. The less stringent criterion evaluated for MIC determination (MICs scored as 2) provided not only better agreement among the laboratories, but a shift in the MIC distribution toward the lower drug concentrations (especially for *C. albicans* and *C. tropicalis*). These low fluconazole MICs (0.2 to 2 μg/ml) also have been reported before when well-defined media were used (9).

The higher agreement of MICs scored as 2 indicated that this less stringent criterion is more reliable for endpoint determinations. This prompted the attempt to quantify these 1 and 2 scores by comparing them with 1:10 and 1:5 dilutions, respectively, of each yeast growth control. Although this experiment was performed after the main study was completed and only with fluconazole against 15 isolates of *Candida* spp., 93% agreement among the four laboratories was found with MICs scored as 2 (MIC 80%). Furthermore, the score 2 corresponded to ≤1:5 growth control dilutions of the yeasts being tested, an estimated 80% decrease in turbidity compared with that of the specific undiluted growth

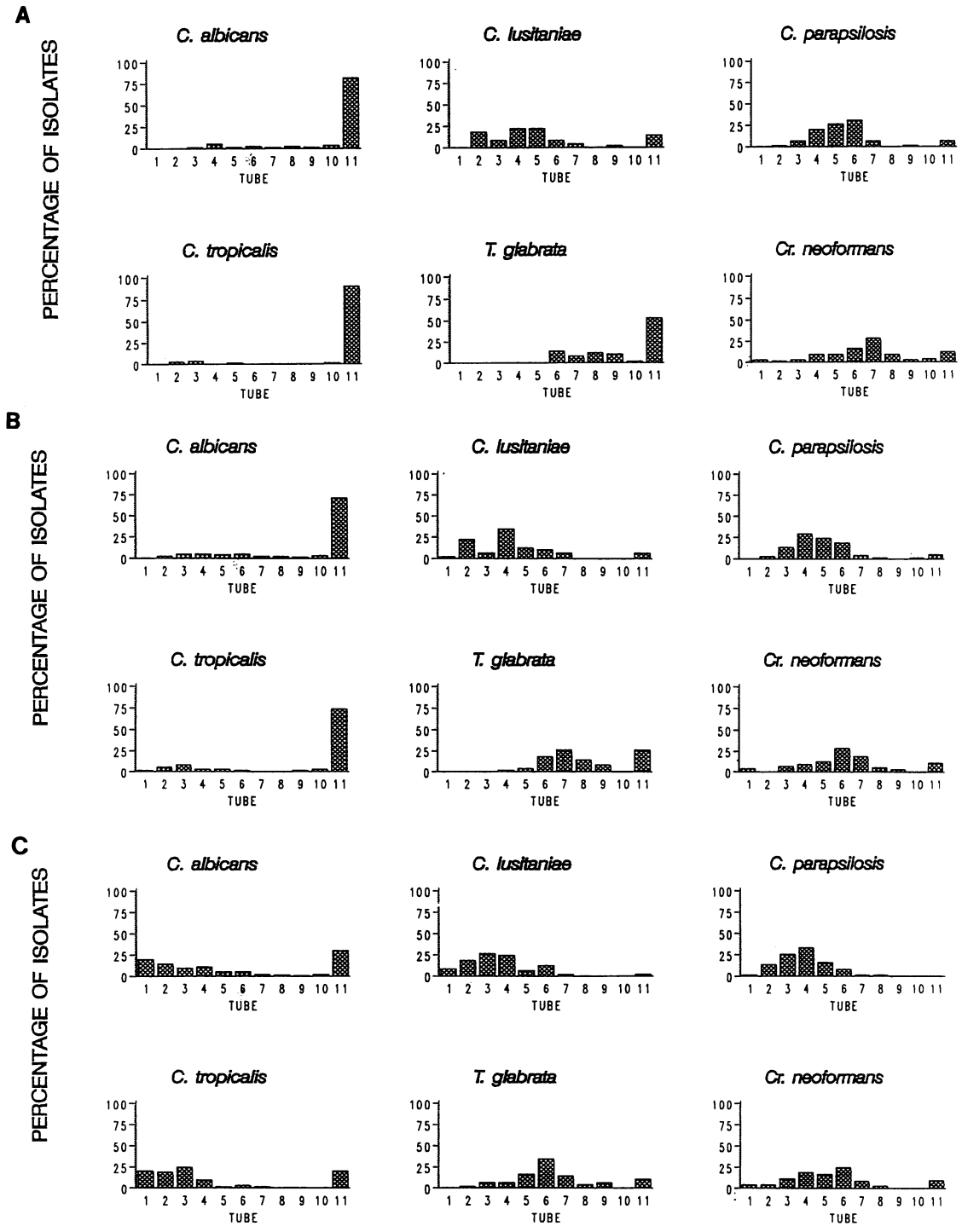

FIG. 1. Broth microdilution distribution of susceptibilities with fluconazole against 95 pathogenic yeast isolates according to three different scoring criteria in five laboratories. MICs were determined with liquid buffered (MOPS) RPMI 1640 medium (pH 7.0), an inoculum density of approximately 0.5×10^3 to 2.5×10^3 CFU/ml, and second-day readings (more than 48 h for *C. neoformans* isolates). Tube numbers indicate drug concentrations, which ranged from 0.12 μg/ml (tube 1) to 64 μg/ml (tube 10) (tube 11, 128 μg/ml). MICs scored as 0 (optically clear) (A), 1 (slightly hazy turbidity) (B), and 2 (prominent decrease in turbidity) (C) are shown.

controls. The dilution value of ≤1:10 (90% decrease in turbidity) corresponded to a score of 1. This convenient and direct method may aid in a more accurate evaluation of each specific MIC endpoint, and it also could reduce the trouble-some subjectivity of MIC determinations. However, more extensive collaborative studies to appraise this method as well as in vivo correlation of these less stringent MICs with the drug efficacy in vivo needs to be conducted.

Reports of recent collaborative MIC studies (10, 19) have demonstrated extreme variability of the susceptibilities of yeasts to all drugs tested (amphotericin B included), which indicated the need for a reference method. In this study, the broth macro- and microdilution MIC data demonstrated good reproducibility (84 to 99%) with amphotericin B with all parameters, with flucytosine MICs scored as 1 and 2 and with fluconazole MICs scored as 2 on day 1. The data for ketoconazole were more variable; however, reproducibility was ≥80% for second-day MICs scored as either 0 or 1 (Table 1). Therefore, a less stringent criterion provided more reproducible results for the 95 yeast isolates tested.

It has been reported that discrepancies between the broth macro- and microdilution tests were not statistically significant (6). Our study demonstrated good intralaboratory agreement in most laboratories using a low-density inoculum and MICs scored as 1 and 2 (lower for 2), with the exception of the azoles. For these drugs, the agreement between the two tests was more variable (Table 2). Since the discrepancies between the two tests were essentially low and the reproducibility of the microdilution MICs was higher than that of the macrodilution MICs of some drugs, the broth microdilution test provided consistent results in this study. This was reinforced by the agreement of the susceptibilities to the four drugs against the QC isolate in each of the numerous sets of experiments performed (Table 3).

Radetsky et al. (24) and other investigators (6) have found that the broth microdilution test with RPMI 1640 medium produced reproducible MIC results. This test has been found to be more efficient and economical and easier to perform than any of the other tests, including the agar dilution test (11). We agree with the latter researchers. This study suggests that the microdilution test is an adequate tool for antifungal susceptibility testing in the clinical laboratory when performed by following the NCCLS preliminary standards for macrodilution testing of yeasts.

In conclusion, this study confirms the progress that has been accomplished toward the standardization of antifungal susceptibility testing of yeasts. It appears that the optimal testing conditions are low inoculum densities (around 10^3 CFU/ml), second-day readings (more than 48 h of incubation time for some *C. neoformans* strains), and an MIC determination criterion (scored as 2) less stringent than those presently used in most laboratories. Our study also suggests that antifungal susceptibility testing may be performed by the broth microdilution test as well as by the broth macrodilution test. However, more studies are needed in order to better define MIC endpoints, especially those of the azoles, and to evaluate in vitro and in vivo correlations of drug efficacy.

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